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PAPER

OEI800 polyconjugates linked with ketalized glycolic acid for use as gene vectors†

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Endosomal escape of DNA polyplexes is one prominent bottleneck involved in the transfection process. Purposely against the low pH level in the endosome compartment, a series of acid-cleavable gene vectors constructed from oligoethyleneimine OEI800 polyconjugates linked with ketalized glycolic acid were designed herein and termed OEI-GKs. Their potential as gene vectors was comparatively evaluated by investigating the properties including DNA binding ability, polyplexes zeta potential, particle size, acid-triggered degradation, buffer capability before and after degradation, cytotoxicity, and transfection efficiency. The resultant data indicate that the transfection efficiency and cell-biocompatibility are dependent on the polymer architecture and molecular weights, which can be tailored by adjusting the charge ratio of OEI800 *versus* the linking agent. OEI-GK(1 : 1) can be potentially developed as efficient vectors for the gene delivery in terms of their transfection activity even higher than PEI25k as well as the negligible cytotoxicity. Those improved properties are believed to have association with ketal-associated degradation of OEI-GK under acid conditions in the endosome, which lead to not only easy unpacking of DNA from the hydrolyzed polyplexes but also, interestingly, substantially enhanced buffer capability.

Introduction

As one important kind of promising gene vector, polycations have attracted great attention in recent years due to many advantages including facile preparation, large payload, tolerance towards gene sizes and low immunogenic risk as compared to viral vectors.[1,2] Although steady developments have been achieved in this field, polycation vectors continue to be optimized to acquire promoted transfection activity and reduced cytotoxicity.

Up to date, the commercially available branched polyethyleneimine (PEI) with a molar mass of 25 kDa (PEI25k) is still regarded as one of the most effective non-viral vectors, and often acts as a "golden standard" to evaluate other newly designed polymeric vectors.[3,4] Unfortunately, its rather high cytotoxicity hampered the application in human gene therapy trials. In contrast to it, low molecular weight oligoethyleneimines (OEI) such as OEI800 (M_w = 800 Da) only display poor transfection activity despite their negligible cytotoxicity.[5–7] Against this catch-22 problem, one reasonable resolution has been proposed by coupling short PEI chains into one entity using biodegradable linkers. This strategy has been partly validated by studies where ester or disulfide cross-linked OEI polyconjugates could mediate modest transfection whilst holding improved cytotoxicity.[8–10] Furthermore, it is expected that those polyconjugates would ultimately break down into OEIs and be readily excreted from the body, leading to a long-term safety profile.

The gene transfection process consists of several biological steps including extracellular and intracellular transfer, vector unpacking as well as the final gene expression in the nucleus. After endocytosis, endosomal escape and following dissociation of nucleic acids from vectors have been recently disclosed to be the crucial efficiency-determining steps.[11,12] As is well-established, polycations could provide tight package and efficient protection of DNA from undesired interaction, and mediate its uptake into the cells *via* electrostatic interaction.[13] Nevertheless, such a strong interaction may be unfavorably a limiting factor for the intracellular transfer process. It would lead to difficult DNA detachment from vectors, inhibitory access by the transcription machinery and consequently low transfection efficiency.[14] At this point, a satisfactory gene delivery system may preferably be constructed *via* the realization of multifunction that maintains extracellular stability of polymer/DNA polyplexes whilst allowing the endosomal escape and intracellular disassembly of polyplexes with relatively little difficulty, in order for the DNA to be released into the cytoplasmic and nuclear sites and become biologically active.[4]

A future aim for gene delivery will be the development of "smart" polyplexes termed artificial viruses.[15] Those polyplexes

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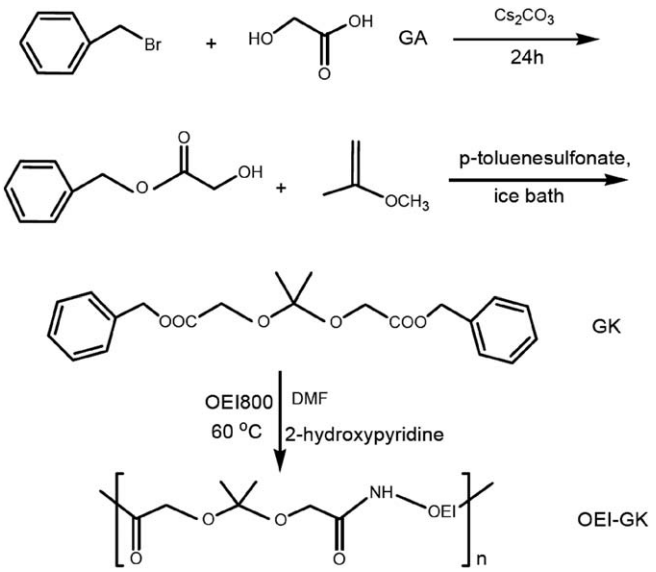
could sense the biological environments and subsequently undergo programmed changes, which would make them more compatible at each step during the transfection.[16] In comparison to the extracellular milieus, the endosomal environment is quite different, where the pH is from about 7.4 in the early period to 5.0 in the late stage.[11] According to such specific pH gradients, many efforts have been devoted to the design of pH-dependent gene vectors, which can generally undergo pH-responsive changes regarding the charge, phase and conformation to provide a membrane-disruptive driving force, for instance the "proton sponge" effect.[17–20] The proton sponge nature of PEI is thought to cause osmotic swelling and physical disruption of the endosome, thus resulting in the escape of the vector from the degradable lysosomal trafficking pathway.[20] Also, such an effect can possibly destroy the pH gradient that in turn disturbs endosome maturation and function. We hypothesize that if OEI polyconjugates decompose into OEI fragments triggered by the local acid environment in the endosome, the membrane-disruptive capability might be enhanced due to the enhanced water-solubility after degradation as well as the colligative feature of the osmotic pressure, possibly leading to a strengthened "proton sponge" effect. Furthermore, the decomposition of those polyconjugates would somewhat lessen the interaction between the vector and DNA, which would help free the condensed DNA for the following transfer process. Such a strategy may also permit the gene delivery system with high gene expression in solid tumor tissues since the local pH levels are lower than those in blood or other normal tissues.[21]

Given those considerations, the design of acid-cleavable OEI polyconjugates might be attractive for the purpose of improved transfection performance. Nevertheless, the relevant researches are rarely reported.[22–24] Wagner's group has recently done pioneering attempts by introducing acetal or ketal groups into the linking reagents.[22] Those acid-sensitively degradable polyconjugates showed enhanced transfection efficacy and biocompatibility. Note that in addition to the acid-cleavable groups, those designed linkers contained ester or maleimido groups which are subject to serious aminolysis reaction with PEI.[3,25,26] Hence, low preparation (below 25 °C) and storage temperatures (−80 °C) together with a short reaction time (45 min) were required in order to abate the side reactions to a certain extent. Even so, the side reaction eventually led to a large molecular weight distribution of the obtained polyconjugates ($M_w/M_n > 4$), incomplete polyconjugate degradation, as well as difficulties in yielding compositionally and architecturally consistent products. It may also raise confusion about the function of endosome-specific degradation because the improved properties appeared to have strong association with the significant amide linkage originating from the side reaction.

In the current study, a novel polycation vector composed of OEI800 units linked *via* an acid-cleavable ketal linkage was fabricated but excluding any amine-labile groups. By design, the linker reagent is derived from glycolic acid (Scheme 1), which can be metabolized *in vivo* and has been widely utilized to prepare biomedical materials such as poly(glycolic acid) (PGA) and poly(lactic-*co*-glycolic acid) (PLGA). The prepared polyconjugates termed OEI-GKs are expected to ultimately degrade into OEI800 and GA *in vivo*, resulting in high biocompatibility.



Scheme 1 Illustration of the syntheses of OEI-GKs.

Experimental section

Materials

Branched PEI25k and PEI800 were purchased from Sigma-Aldrich. Glycolic acid, benzyl bromide, 2-methoxypropene, *N*,*N*-dimethylformamide (DMF) and petroleum ether were obtained from Shanghai Chemical Reagent Co. (China). DMF was dried under CaH_2 before use. QIAfilter™ plasmid purification Giga Kit (5) was purchased from Qiagen (Hilden, Germany) and GelRed™ was purchased from Biotium (CA, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), Hoechst 33258 and Dulbecco's phosphate buffered saline (PBS), penicillin-streptomycin, trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Invitrogen Corp. A Micro BCA protein assay kit was obtained from Pierce. Other reagents were analytical grade and used as received.

Synthesis of 2-hydroxylbenzyl acetate

2-Hydroxylbenzyl acetate was prepared according to the reference.[27] 10 g of Cs_2CO_3 dispersed in 40 mL H_2O was dropped into a mixed solvent of MeOH and H_2O (20 : 3, v/v, 115 mL) containing 5 g of glycolic acid. The solution was then neutralized. After stirring for 10 min at room temperature, the solvent was thoroughly removed under reduced pressure and the residual was dissolved in 150 mL DMF. Benzyl bromide (8 mL, 67.5 mmol) was added into that solution slowly. After stirring at room temperature for 24 h, the reaction was quenched by saturated NaCl solution. The solution was extracted with EtOAc (3 × 40 mL). After drying over $MgSO_4$ followed by concentration, the product was purified by column chromatography (EtOAc : petroleum ether = 1 : 4).

Synthesis of benzyl-2,2′-(propane-2,2-diylbis(oxy)) diacetate (GK)

According an amended method,[28] using an ice bath, 2-methoxypropene (1.015 mL, 20 mmol) was slowly dropped to

2-hydroxylbenzyl acetate (3.5192 g, 20 mmol), which was firstly suspended in 80 mL of dry petroleum ether containing *p*-toluenesulfonic acid (38 mg, 0.4 mmol). After stirring for 2 h, another 20 mmol of 2-methoxypropene was added. The reaction process was monitored by thin layer chromatography (TLC). Upon completion of the reaction, 6 mL of triethylamine was added to neutralize the *p*-toluenesulfonic acid and the product was purified by column chromatography (EtOAc : petroleum ether = 1 : 6).

Syntheses of OEI-GKs

An appropriate amount of benzyl-2,2′-(propane-2,2-diylbis (oxy)) diacetate was dissolved in DMF at a concentration of 0.1 g mL^{-1}. One molar equivalent of OEI800 (1.6 g mL^{-1}) and two equiv. of 2-hydroxylpyridine were dissolved in DMF. Under stirring at 50 °C, the former solution was slowly dropped into the OEI800 solution. After standing for 48 h, the final product OEI-GK(1 : 1) was collected as yellow oil *via* the precipitation method using petroleum ether as the poor solvent. Likewise, OEI-GK (1 : 2) and OEI-GK(2 : 1) were obtained at different charge ratios in feed. The overall yield was around 80% for all the reactions.

Characterization of OEI-GKs

Fourier transformed infrared (FTIR) spectra were recorded on a Perkin Elmer-2 spectrophotometer. Prior to the measurement, the sample was pressed into potassium bromide (KBr) pellets. ^1H nuclear magnetic resonance (^1H NMR) spectra were recorded on a Varian Mercury-VX 300 spectrometer using D_2O as solvent. Size exclusion chromatography and multi-angle laser light scattering (SEC-MALLS) analysis was utilized to determine the molecular weights of the polymers. A dual detector system, consisting of a MALLS device (DAWN EOS, Wyatt Technology) and an interferometric refractometer (a differential refractive index detector) (Optilab DSP, Wyatt Technology) was used. The polymer concentration was 10 mg mL^{-1}. DMF was used as the eluent for OEI-GK and ultrapure water was used for OEI-GKs at a flow rate of 0.3 mL min^{-1}. The column temperature was fixed at 25 °C and the MALLS detector was operated at a laser wavelength of 690 nm.

Cell culture

African Green Monkey SV40-transfected kidney fibroblast cell line (COS7) and human cervix carcinoma (HeLa) cells were incubated with DMEM containing 1% antibiotics (penicillin-streptomycin, 10 000 U mL^{-1}) and 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

Agarose gel retardation assay

OEI-GKs/pDNA complexes at different w/w ratios were prepared by adding an appropriate volume of OEI-GK solution (in 150 mM NaCl solution) to 0.1 μg of pGL-3. The obtained complex solution was diluted by 150 mM NaCl solution to the total volume of 8 μL and vortexed for 30 s before being incubated at 37 °C for 0.5 h. Then 1 μL GelRed™ was added to the complexes respectively prior to be electrophoresed on the 0.7% (w/v) agarose gel which was put in Tris-acetate (TAE) running buffer at 80 V for 1 h. Finally the complexes were observed by a UV lamp with a Vilber Lourmat imaging system (France).

Amplification and purification of plasmid DNA

pGL-3 was used in the experiment. It was transformed as the luciferase reporter genes in *Escherichia coli* JM109 and amplified in the terrific broth media by acid–base titration at 37 °C overnight. The amplified plasmid was first purified by an EndoFree QiAfilter™ Plasmid Giga Kit (5) then dissolved in TE buffer solution and stored at −20 °C. Finally the purity and concentration of plasmids was determined by ultraviolet (UV) absorbance at 260–280 nm.

In vitro transfection of luciferase assay

Transfection of pGL-3 plasmids mediated by OEI-GKs in COS7 and HeLa cells was studied and PEI25k was used as the positive control. Cells were seeded at the density of 6×10^4 cells/well in the 24-well plate and incubated at 37 °C for 24 h respectively. The complexes at various w/w ratios ranging from 5 to 30 were prepared by adding 1 μg of pGL-3 DNA to an appropriate volume of OEI-GK solution (in 150 mM NaCl solution). Then the polyplexes solution were vortexed for 30 s before being incubated at 37 °C for 0.5 h and then diluted to 1 mL by serum-free DMEM before transfection. After being incubated for 4 h at 37 °C, the serum-free DMEM was replaced by fresh DMEM containing 10% FBS and further incubated at 37 °C for 44 h. The luciferase assay was followed according to manufacture's protocols. Relative light units (RLU) were measured by chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The total protein was determined according to the BCA protein assay kit (Pierce). Luciferase activity was expressed as RLU/mg Protein. Data were shown as mean ± standard deviation (SD) based on three independent measurements.

Confocal laser scanning microscope

To study cellular uptake of the complexes, fluorescent confocal microscopic images of complexes in HeLa cells were further obtained. HeLa cells were seeded at the density of 1×10^5 cells/well in the 24-well plate with 1 mL of DMEM containing 10% FBS and incubated at 37 °C for 24 h. 1 μg of pGL-3 was fluorescently labeled with 5 μL (10×10^{-6} M) YOYO-1 iodide at 37 °C for 15 min before the complexes were added and further incubated for 15 min. Then 900 μL of DMEM containing 10% FBS was added and incubated at 37 °C for 4 h. After that, cells in each well were washed five times with PBS and 40 μL Hoechst (0.2 mM in DMEM containing 10% FBS) was then added and incubated at 37 °C for 0.5 h. Then the cells were photographed by a confocal laser scanning microscope (Nikon C1-si TE2000, Japan) and finally recorded by EZ-C1 software.

Cytotoxicity assay

The cell toxicity of OEI-GKs was examined by MTT assay. The COS7 cells and HeLa cells were seeded in the 96-well plate with a density of 6000 cells/well and cultured 24 h in 100 μL DMEM

containing 10% FBS respectively. After incubating the polymers for 48 h, 20 μL of MTT (5 mg mL^{-1}) solution was added in and further incubated at 37 °C for 4 h. Then the medium was removed and 150 μL DMSO was added in. A microplate reader (BIO-RAD 550) was used to measure the absorbance of the medium at 570 nm. The relative cell viability was calculated as follows:

$$\text{Relative cell viability } (\%) = \frac{(OD_{570,sample} - OD_{570,background})}{(OD_{570,control} - OD_{570,background})} \times 100\%$$

where $OD_{570,sample}$ was obtained in the presence of polymers, $OD_{570,control}$ was obtained in the absence of polymers and $OD_{570,background}$ was obtained in the absence of polymers and cells. Data were shown as mean ± standard deviation (SD) based on triplicate independent experiments.

Degradation test

For the degradation study, polymers were dissolved in solution with different pH values at 5.5 and 7.2 for a period of time. The biodegradation was evaluated by monitoring the DNA binding ability of the degraded polymers using an agarose gel retardation assay. In the buffer capability test, the samples were incubated for 8h before acid–base titration.

Acid–base titration

The buffer capability of samples was evaluated by acid–base titration assay over the pH range from 10.0 to 2.50. Briefly, 0.2 mg mL^{-1} of each sample solution was prepared in 30 mL of 150 mM NaCl solution before titration. Firstly the sample solution was titrated by 0.1 M NaOH to the pH of 10.0. Then 0.1 M HCl was gradually dropped into the solution to obtain mixtures with different pH values which were measured by a microprocessor pH meter simultaneously until the pH value decreased to 2.50. Data were shown as mean ± standard deviation (SD) based on three independent measurements.

Particle size and zeta potential measurement

Particle size and zeta potential were evaluated by dynamic light scattering (DLS) at 25 °C by Nano-ZS ZEN3600 (Malvern Instruments). The complexes at various w/w ratios ranging from 5 to 30 were prepared by adding 1 μg of pGL-3 DNA to an appropriate volume of OEI-GK solution (in 150 mM NaCl solution). Then the complexe solutions were vortexed for 30 s before being incubated at 37 °C for 0.5 h and then diluted to 1 mL by 150 mM NaCl solution prior to being measured. Data were shown as mean ± standard deviation (SD) based on three independent measurements.

Results and discussion

Syntheses and characterization of OEI-GKs

In the present study, ketalized OEI polyconjugate vectors termed OEI-GKs were prepared (Scheme 1), aiming at efficient cytoplasmic localization and relatively easy disassociation of the polyplexes through the hydrolysis of ketal bonds under acid conditions in the endosome. The linking reagent benzyl-2,2′-(propane-2,2-diylbis(oxy)) diacetate (GK) was specifically designed (Scheme 1) starting from glycolic acid, providing long-term safety *in vivo*. GK is constructed around a centrally located ketal bond, and can react with primary amines to form covalently stable linkages. Those molecule designs avoid the possible side-reactions and, thus, a useful model can be established to exclusively investigate the relationship between endosome-associated polymer degradation and gene transfection efficacy.

By adjusting the charge ratio of OEI800 *versus* GK, three OEI-GKs were prepared in DMF (Table 1). As reported, the linking reaction of OEIs would sometimes lead to the formation of insoluble gels.[29] In the present preparation, by slowly introducing the linking reagent into the OEI800 solution, the reaction mixture remained transparent throughout the whole process, even when [GK] : [OEI800] approached 2 : 1. All the products were soluble in water.

The chemical structures of OEI-GKs were identified by IR† and ^{1}H NMR analyses (Fig. 1). All the characteristic resonances from the GK linker and OEI800 moieties are clearly detectable. In the IR spectra, a strong absorbance of carbonyl groups ($\nu_{C=O}$) appears at 1662 cm^{-1} and the peak at 1386 cm^{-1} is attributed to the stretching frequency of amide C–N (ν_{C-N}) (Fig. S1†). As shown in the ^{1}H NMR spectrum of OEI-GK(1 : 1) (Fig. 1B), besides the characteristic signals in the region δ 2.49–2.61 ppm (signal **e**) belonging to OEI800, two separate single peaks at δ 3.90 ppm (signal **b**) and at δ1.30 ppm (signal **a**) were clearly detectable, which should be attributed to the $–COCH_2–$ and $–CH_3–$ in GK, respectively. Moreover, it is observed that the signals from the benzyl group of GK were almost lost after the linking reaction, suggesting that the reaction was near completion under the given conditions. By comparing the area integrals between signal **b** and **a**, it could be concluded that nearly no or insignificant hydrolysis of the ketal groups took place throughout the preparation process. Taken together, the spectroscopic analyses strongly demonstrate that by our method, OEI-GKs have been successfully prepared.

The molecular weights of OEI-GK products purified by the precipitation method were determined by SEC-MALLS and the resultant data were collected in Table 1. For the polycondensation between OEI and GK, the molar feed ratio would affect not only the molecular weights but also the polymer architectures, which are well known to play an important role in the transfection process.[1,2] Provided that OEI800 can be taken for a special repeat unit, according to the feed ratio, OEI-GK (1 : 2) could be viewed as a polyconjugate adopting the relatively more branched structure due to the presence of excessive NH_2 groups in PEI.[30] By contrast, we can approximately look on OEI-GK(1 : 1) and OEI-GK(2 : 1) as linear alternative copolymers composed of OEI800 and GK units with different chain lengths.

In vitro cytotoxicity

The biocompatibility is one critical prerequisite of gene vectors for *in vivo* application. Although high molecular weight PEI such as PEI25k displays high transfection efficiency towards most cell lines, its clinical application appears to hardly be realized unless the toxicity issues can be addressed.

Table 1 Reaction condition and results of OEI-GK preparations

Sample	Feed molar ratio (OEI/GK)	Composition (OEI/GK)[a]	M_w/g mol^{-1} [b]	PDI (M_w/M_n)[b]
OEI-GK(1 : 1)	1 : 1	1 : 1	21 300	1.10
OEI-GK(1 : 2)	1 : 2	1 : 2.1	30 500	1.02
OEI-GK(2 : 1)	2 : 1	1.3 : 1	9400	1.47

[a] Data were determined by ^{1}H NMR analyses. [b] Data were obtained from SEC-MALLS analyses.

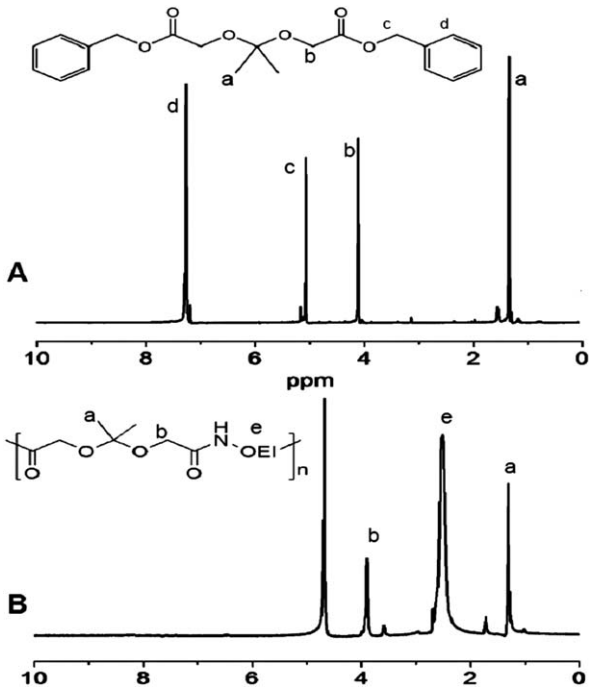


Fig. 1 ^{1}H NMR spectra of GK linker and OEI-GK(1 : 1).

Many studies have revealed that the free polycations rather than the bonded polycations are indeed the major cause of the acute toxicity of the polycation/DNA gene transfer system.[31] In order to preliminarily evaluate the safety profiles of OEI-GKs, *in vitro* cytotoxicity tests were conducted in COS7 and HeLa cells by MTT assay using PEI25k and OEI800 as the control. Fig. 2 shows that OEI-GKs exhibited a remarkably lower cytotoxicity than PEI25k in two different cell lines. For one specified cationic polymer, the cytotoxicity is suggested to be usually proportional to the molecular weights.[6,7] Thus the highest cell-biocompatibility of OEI-GK(2 : 1) can be understood as a consequence of its lowest molecular weight. Both OEI-GK(2 : 1) and OEI-GK(1 : 1) displayed better or comparable cell-biocompatibility compared to OEI800. Relatively, OEI-GK(1 : 2) presented slightly higher cytotoxicity. Due to the relatively more branched and compact architecture, OEI-GK(1 : 2) may possess a larger volume charge density. In comparison, linear OEI-GK(1 : 1) adopts a relatively flexible conformation and the positive charges are more dispersed. Possibly owing to such structural difference, OEI-GK(1 : 2) displayed a higher cytotoxicity despite the relatively lower amine contents. Even so, the viabilities of both cells after co-incubation with OEI-GK(1 : 2) were dramatically higher than those with PEI25k. Specifically, no IC_{50} can be detected throughout the OEI-GK(1 : 2) working concentrations up to 800 μg mL^{-1}. In sharp contrast to this, 80% cells have died at a concentration as small as 50 μg mL^{-1} in the case of PEI25k. Such a marked deviation in cell-biocompatibility between OEI-GK(1 : 2) and PEI25k, despite their similar structures and the higher M_w of OEI-GK(1 : 2), may be attributed to the lower amine content of the former as well as its biodegradation into smaller fragments.[7] The above results clearly indicate that OEI-GKs designed in the present study are much safer than PEI25k for *in vivo* application, and can be used in a broad concentration range.

In addition to the acute toxicity aspect, the long-term fate of the polymeric carrier has to be considered in an organism. Therefore, implanted biomaterials, if they can be degradable *in vivo*, would be more advantageous. OEI-GK vectors are expected to ultimately degrade *in vivo* into low toxicity components, providing a reasonably long-term safety profile.

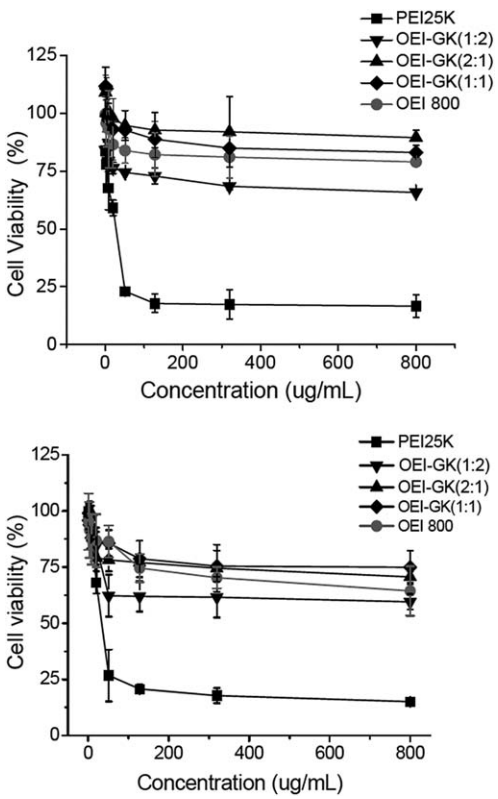


Fig. 2 Cytotoxicity of OEI-GKs in COS7 cells (top) and HeLa cells (bottom) in comparison to that of PEI25k and OEI800. Data were shown as mean ± SD ($n = 3$).

DNA binding assay

Binding with DNA is one of the most important properties for cationic vectors to provide an effective payload and protection of DNA from DNase digestion. An agarose gel retardation assay was performed to investigate whether the synthesized OEI-GKs were able to condense pGL-3 plasmids. As shown in Fig. 3, the mobility of the pGL-3 bands was gradually retarded as the w/w ratio of OEI-GKs/DNA increased. The lowest retarding ratio, where the mobility of pGL-3 could be entirely retarded, was used as the indicator to evaluate the binding ability of the investigated polymers. It is found that the binding ability is dependent on the composition of the OEI-GKs. Amongst the three vectors, OEI-GK(1 : 1) displayed the significantly strongest binding strength as reflected by the rather low retarding ratio at 0.8. OEI-GK (2 : 1) in the case of the ratio at 30 could barely inhibit pGL-3 migration in agarose gel electrophoresis. The capability of OEI-GK(1 : 2) to integrate with plasmid DNA fell between those two.

The higher amine content of OEI-GK(1 : 1) may be a main factor to account for its higher DNA binding ability as compared to the case of OEI-GK(1 : 2). As for OEI-GK(2 : 1), the poor binding ability should be attributed to its lower molecular weight. In addition, the architecture of the obtained polyconjugates may somewhat affect the binding between DNA and polymers. Relatively, linear OEI-GK(1 : 1) is more flexible and thus more OEI moieties per OEI-GK molecule could readily access the DNA as compared to the hyperbranched OEI-GK(1 : 2), which allows compact condensation at a lower retarding ratio.

Particle size of OEI-GK polyplexes

To mediate endocytosis through a cell membrane, cationic polymers need to condense DNA into compact particles. The particle size of polycation/DNA polyplexes is assumed to be an important factor for the achievement of efficient transfection.[32] In the current study, the dynamic light scattering technique (DLS) was utilized to measure the size of the polyplexes at different w/w ratios and the pGL-3 gene was used as the reporter gene.

As seen from Fig. 4, the particle sizes of OEI-GK(1 : 1) polyplexes seemed unchanged irrespective of the measured w/w ratios ranging from 5 to 50. In comparison, after the ratio reached about 20 for OEI-GK(1 : 2) and 30 for OEI-GK(2 : 1), the size of the polyplexes tends to level off. Generally, those results correlate well with the condensation efficiency of OEI-GKs revealed by the DNA binding assay. When the negatively charged DNAs were almost completely complexed and condensed by the positively charged OEI-GKs, further increase in the w/w ratio would exert an insignificant effect on the particle size. Relatively, OEI-GK(1 : 1)/DNA polyplexes presented a smaller particle size than the other two polyplexes. This can be explained as the consequence of its stronger DNA binding ability. The polyplexes formed from OEI-GK(2 : 1) may be of a loose structure due to the weak vector/DNA interaction, leading to the largest particle size among three investigated polyplexes. As for branched OEI-GK(1 : 2) polyplexes, in addition to the effect of binding ability, the strong steric hindrance might also confer the relatively larger particle size.

All of the polyplexes measured had particle sizes above 350 nm. Although nano-sized polyplexes at around 100∼150 nm are assumed to be advantageous for cellular uptake through non-receptor-mediated endocytosis, this opinion is still debatable.[32] The opposing results have shown that some larger particles could mediate a higher transfection efficiency than smaller particles.[33] From another viewpoint, the large particle size of the OEI-GK polyplexes, to some extent, implies the relatively loose structure within the polyplexes. Properly loosening the polyplex structure may be beneficial for the improved gene transfection since it would facilitate the easy approach of transcription-factors to the swollen polyplexes. Recent studies have also revealed that in both polyplexes and liposomes, the plasmid DNA needs to be freely accessible for cell membrane interaction.[7,14] Those reported findings suggest that the particle size may not be the primary factor determining the transfection efficacy.

Zeta potential of OEI-GK/DNA polyplexes

Effective gene transfection mediated by polycation vectors depends upon its ability to condense DNA into polyplexes with positive charges, so as to easily access the negatively charged cell

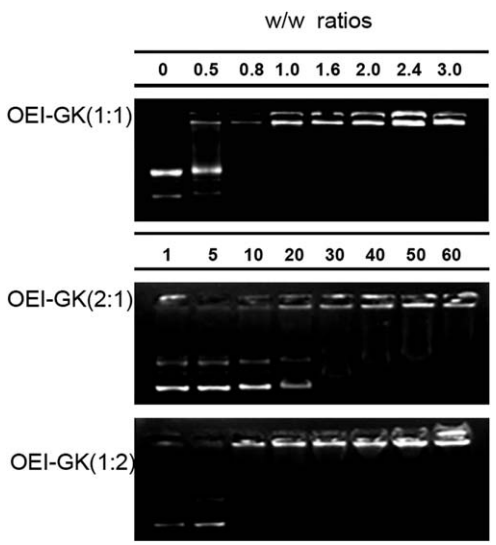


Fig. 3 Agarose gel electrophoresis retardation assay of OEI-GKs.

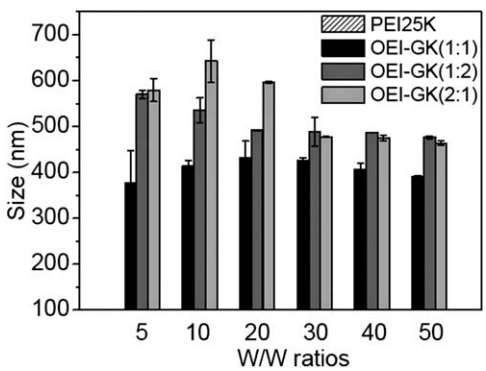


Fig. 4 Particle size of OEI-GK/DNA polyplexes at different w/w ratios. Data were shown as mean ± SD ($n = 3$).

membrane and promote the endocytosis by increasing either the uptake rate or the endocytosis amount.[34,35] Nevertheless, an excessively high surface charge would lead to a detrimental effect on the cells, which results in a compromised transfection efficacy.

Similar to the analyses of polyplex particle sizes, the surface charges of the OEI-GK polyplexes also largely rely on the polymer composition and architecture (Fig. 5). OEI-GK(1 : 2) showed an apparently higher zeta potential than the other two polyplexes, presumably due to more bound polymers per pDNA and/or high volume charge density associated with the highly branched structure. Note that although the binding assay indicates that the DNA migration could be completely retarded at the w/w ratio above 30 for OEI-GK(2 : 1), the polyplexes in the case of relatively lower w/w ratios possessed positive charges. It suggests that the formed polyplexes of OEI-GK(2 : 1) are not stable, which somewhat agrees with their larger particle size as measured by DLS.

Binding ability assay after acid-triggered degradation

Viral vectors typically represent environment-responsive metastable structures that simultaneously possess the stability to withstand environmental damages as well as the instability to respond to environmental cues, thus releasing the nucleic acid at the appropriate sites.[15,36] For bio-simulation purposes, polyplexes had better respond in an "intelligently dynamic" manner to their microenvironment and in turn trigger correspondingly adaptable functions at transfer steps. Responding to the unique pH gradients in an endosome,[11] the cleavage of ketal bonds in OEI-GKs can be locally acid-triggered, leading to the partial decomposition of OEI-GK polyconjugates into smaller fragments. In addition to the expected long-term safety, such biodegradation may also affect the binding ability and membrane-disruptive capability as discussed below.

OEI-GK(1 : 1) was chosen as the model to identify the pH-responsive biodegradation and the resulting decline of DNA binding ability. The hydrolyses of OEI-GK(1 : 1) in endosomal and physiological environments were simulated by incubating the polymer in PBS with different pH values of 7.2 and 5.5, respectively. After the treatment, the vectors were subjected to agarose gel electrophoresis assay to investigate the DNA binding ability (Fig. 6).

At pH = 7.2, OEI-GK(1 : 1)/DNA polyplexes are disclosed to possess reasonably good stability as can be evidenced by slight increment of the retarding ratio even after 48 h of incubation (Fig. 6). Such structural stability of polyplexes is advantageous for the protection of DNA from DNase degradation before entering into cells. In sharp contrast to it, upon exposure to PBS solution at pH = 5.5, a marked decline of the binding strength was distinctly observed. And the decline of DNA binding ability was presented more profoundly as prolonging the incubation time. It is noted that at pH = 5.5, the protonated OEI-GK should indeed have a stronger binding ability than its unprotonated counterpart due to the higher charge density, provided that no degradation occurs. Evidently, such an apparently reduced affinity for DNA should be attributed to the acid-triggered cleavage of ketal bonds, resulting in the formation of low molecular weight OEI-GK fragments.

Those results suggest that the condensed DNA may be relatively easily released from OEI-GK polyplexes after the acid-triggered degradation in endosome.[37] Lauffenburger and co-workers established a computational model for intracellular gene delivery, indicating that rather low dissociation rates of polyplexes would lead to low levels of transfer expression.[38] Although the appropriate interaction strength between DNA and vector for the intracellular transfection process is not yet clear, it is assumed that the partial decomposition of OEI-GK(1 : 1) and the consequently reduced DNA–vector interaction occurring at the endosome-escape step, may help free the DNA from the hydrolyzed polyplexes and benefit the following transfection procedures.

Comparison on the buffer capability before and after degradation

Endosomal escape is considered as one critical barrier with regard to the efficient delivery of nucleic acids to cytoplasmic and nuclear sites. Unlike viral vectors having fusion peptides at their surface and being capable of escape from the endosome by membrane fusion, the buffer capability of PEI gives rise to the so-called "proton sponge" effect, which is assumed to be triggered by an osmotic imbalance of the endosome upon PEI protonation.[20] The effect is believed to contribute much to the destabilization of the endosome and subsequent cytoplasmic release of internalized polyplexes. Therefore, the buffer capability can act

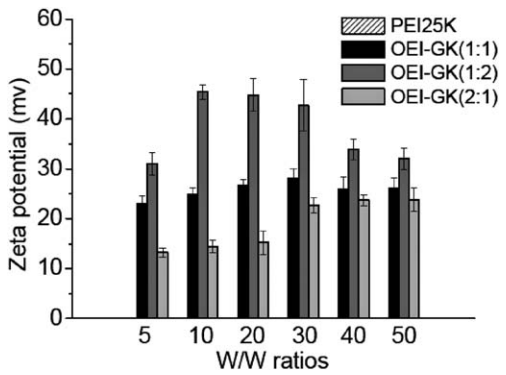


Fig. 5 Zeta potential of OEI-GK/pDNA polyplexes at different w/w ratios. Data were shown as mean ± SD ($n = 3$).

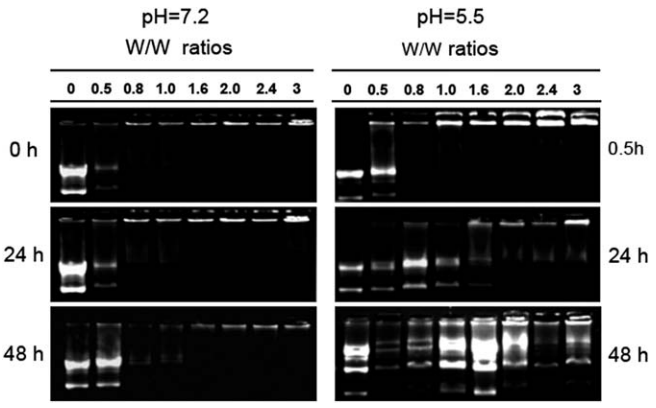


Fig. 6 Agarose gel electrophoresis assay of OEI-GK(1 : 1) at different pH values.

as a useful indicator to roughly reflect the strength of the "proton sponge" effect.

In the present work, acid–base titration was used to evaluate the buffer capability. The concentration of OEI800 moieties was identical for all the investigated polyconjugate solutions before titration. As shown in Fig. 7A, the buffer capability of the OEI-GKs dropped substantially as compared to OEI800. Such a decline of the buffer capability is usually reported for the modified PEI derivatives and is attributed to the loss of free amino groups after modification. Nevertheless, as seen in Fig. 7B, upon acid-triggered degradation, OEI-GK(1 : 1) and OEI-GK(2 : 1) exhibited insignificant difference in the titration curve compared with OEI800. The interesting restoration of buffer capability can be ascribed to the degradation of OEI-GKs into small fragments with much better water-solubility. We thus propose that the decline of buffer capability upon GK linking should be more possibly associated with the decreased water-solubility after polyconjugation. This explanation can be demonstrated by the fact that alkylated PEI derivatives modified with longer alkyl chains showed a relatively weaker buffer capability despite the same substitution degrees.[39] For this reason, OEI-GK(2 : 1) with lowest molecular weight but better water-solubility displayed much better buffer capability whereas the other two were almost identical.

The results suggest that the membrane-disruptive capability would be greatly enhanced after OEI-GK degradation. This degradation-associated change agrees somewhat with the proposed conception termed "masked endosomolysis", where the pH-labile bonds were designed to mask endosomolytic agents but would undergo chemical bond cleavage to unmask a compound's endosomolytic activity.[40–42] Such an 'intelligent' mask would permit efficient *in vivo* delivery without sacrificing endosomolytic function for release into the cytoplasm. In addition, the strong buffer capability would prevents the acidification of endosomes and their fusion with lysosomes, by which the possible enzymatic degradation of DNA is retarded.

Another important finding is noted that although the buffer capability of OEI-GK(1 : 2) had an apparent promotion after degradation relative to the raw polymer prior to degradation, it was still lower than that of OEI800. This implies that the linear OEI-GK(1 : 1) may be more sensitive to acid-triggered degradation than OEI-GK(1 : 2) with a three-dimensional hyperbranched architecture.[43] Possibly, the ketal groups in linear OEI-GKs with a relatively flexible conformation in solution are easily exposed to the exterior acid environment, leading to a faster degradation rate.

In vitro transfection

The transfection mediated by OEI-GKs was investigated in COS7 and HeLa cells, respectively. In the luciferase assay, plasmid pGL-3 served as the reporter gene, and PEI25k, well-established as the golden standard, was used as the control for comparison purpose at its optimal w/w ratio of 1.3.[3]

As clearly observed from Fig. 8, OEI-GK(1 : 1)/DNA polyplexes displayed the highest transfection efficiency amongst the

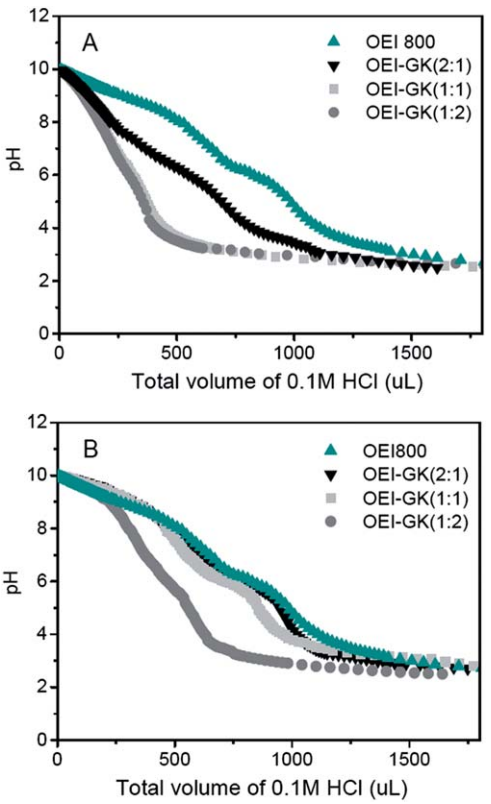


Fig. 7 Acid–base titration profiles of OEI800 and OEI-GKs before (A) and after degradation (B) in 0.15 M NaCl solution. Data were shown as mean ± SD ($n = 3$).

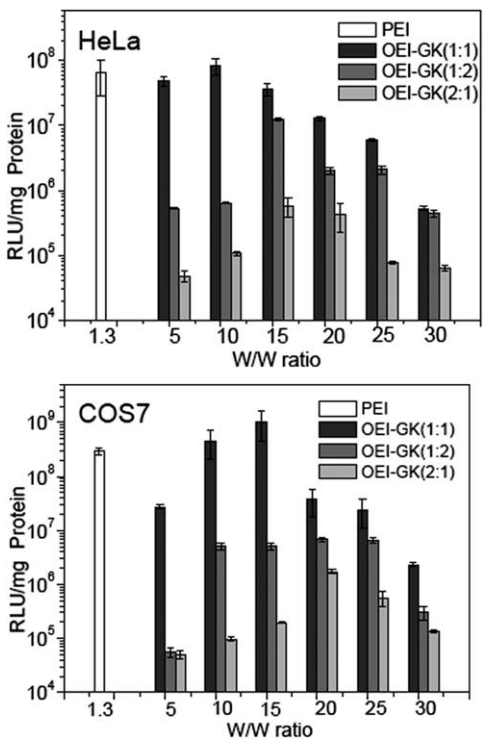


Fig. 8 Transfection efficiency of OEI-GKs/pDNA polyplexes at different w/w ratios in COS7 cells (bottom) and HeLa cells (top) compared with PEI25k control at w/w 1.3. Data were shown as mean ± SD ($n = 3$).

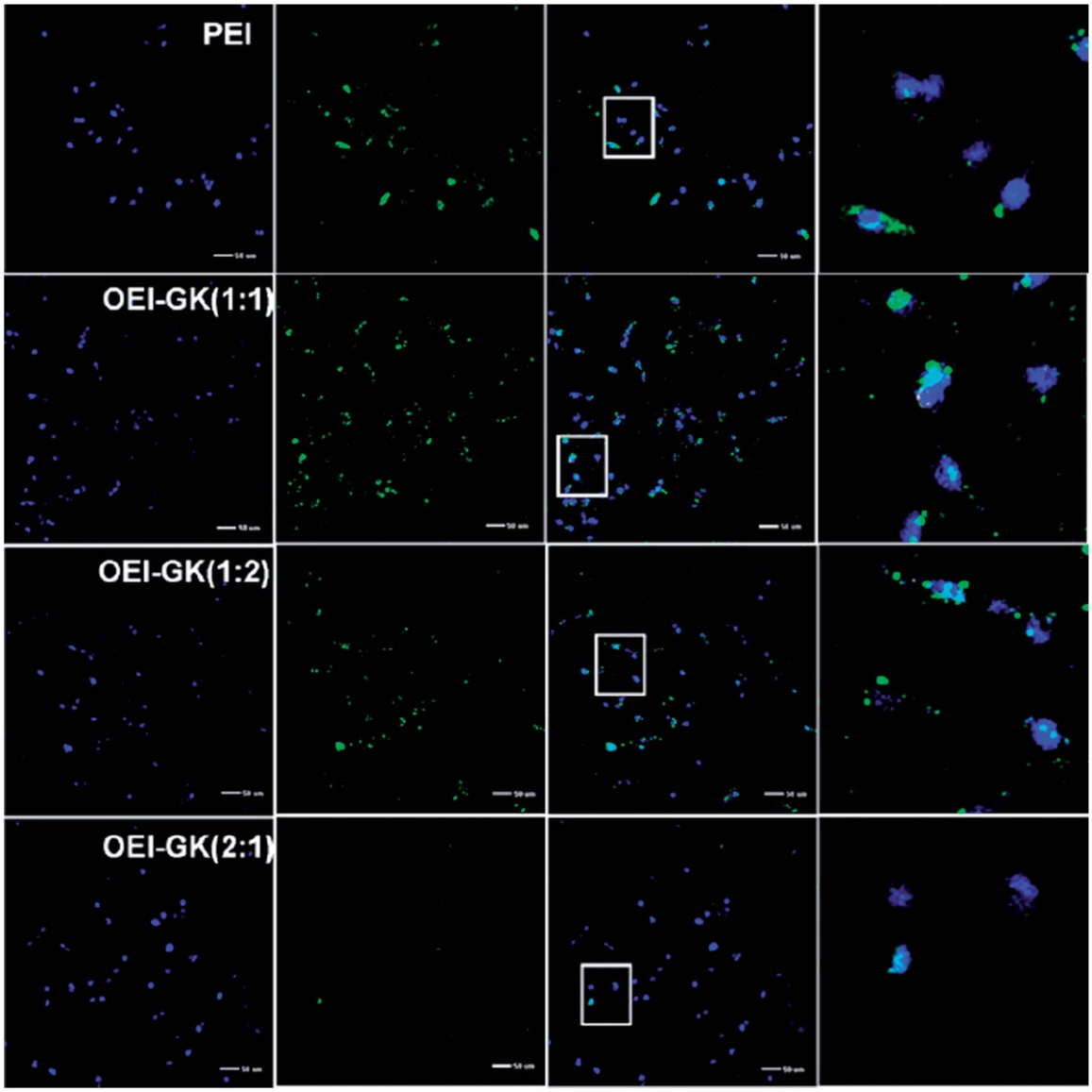


Fig. 9 Fluorescent confocal microscopic images of OEI-GK/pGL-3 distribution in HeLa cells.

three OEI-GK polyplexes in both cell lines. At the w/w ratio of about 10∼15, the maximal luciferase expression was observed in two cell lines transfected by OEI-GK(1 : 1)/DNA polyplexes. OEI-GK(1 : 1) can mediate the transfection at the level even higher than that of PEI25k by a factor of up to 10. Condensation of DNA by a gene vector plays a key role in determining the extracellular stability, endocytosis of polyplexes, and final gene expression.[44] OEI-GK(2 : 1)/DNA polyplexes were the least effective in gene transfection despite having the highest cell-biocompatibility, possibly due to the rather low DNA binding ability. Hypothetically, there is a possibility that OEI-GK(2 : 1)/DNA may potentially act as the vectors for the siRNA delivery in terms of the much smaller size of siRNA and the need for only cytoplasmic release as compared to pDNA delivery.[45]

Provided that the higher transfection activity of OEI-GK(1 : 1) over PEI25k is attributed to the lower cytotoxicity and the degradation-induced structural destabilization of the polyplexes,[46] it would be difficult to understand why OEI-GK(1 : 2) with a similar structure to PEI25k did not display the similarly high transfection activity as anticipated in spite of its higher molecular weight. Possibly, in addition to the buffer capability for endosomal escape, the need for proper DNA binding ability before and after degradation (responding to extracellular and intracellular transfer) should be considered to account for this finding.

Overall, the luciferase assay suggests that the rational structural control on OEI-based polyconjugate vectors is of great importance for a desirable transfection performance. It appears that the architecture of OEI-GK(1 : 1) combining the branched structure of OEI and the linear structure adopted by OEI-GK (1 : 1) is superior to the thoroughly hyperbranched structure of OEI-GK(1 : 2).

Intracellular fate of DNA/OEI-GK polyplexes

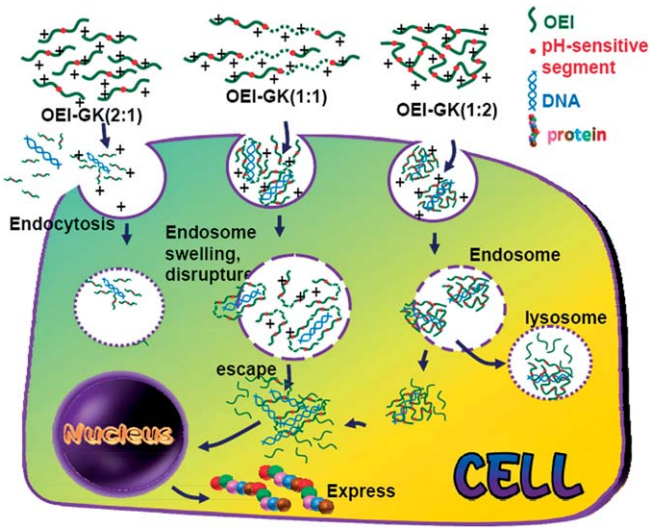
To visualize the intracellular localization of DNA after cellular uptake, cancerous HeLa cells were incubated with pGL-3

polyplexes at 37 °C for 4 h and then observed by confocal laser scanning microscopy. The pGL-3 plasmids in polyplexes were labeled with YOYO-1 iodide, presenting green fluorescence. To obtain further information concerning the cellular uptake, the nuclei were stained by Hoechst 33258 and presented blue to distinguish with the green fluorescence.

Tracking the DNA/OEI-GKs provides images similar to those observed for PEI25k/DNA polyplexes (Fig. 9). Indeed, green pGL-3 plasmids appear as discrete spots located in the cytoplasm and blue-labeled nuclear areas. In comparison with other vectors, OEI-GK(1 : 1) was evidently more effective in transporting pGL-3 plasmids into the nuclei in terms of the stronger florescence intensity. In contrast, very small amounts of green spots could be detectable in the cells transfected with OEI-GK (2 : 1) polyplexes. This indicates that the OEI-GK(2 : 1) polyplexes are difficult to enter into cells due to the instability of the polyplexes leading to premature release and degradation of DNA outside the cells. All the observations were in quite good consistency with the quantitative analyses by luciferase assay.

Hypothesis regarding OEI-GKs mediated transfection

Regarding the OEI-GKs mediated transfection, it is believed that several main factors should be comprehensively taken into account including the degradation rate of OEI-GKs, the DNA binding ability and the buffer capability before and after degradation. Based on all the results obtained in this study, we may explain the difference in the transfection efficiency among OEI-GKs in such a way as illustrated in Scheme 2. Due to the low binding ability, the premature extracellular release of DNA is the main cause for the low transfection efficiency of OEI-GK(2 : 1). As for OEI-GK(1 : 1), the strong DNA binding ability confers extracellular protection of DNA and effective endocytosis into the cells. At the same time, acid-triggered degradation in the endosome after endocytosis could lead to substantially enhanced buffer capability and a somewhat reduced interaction between DNA and vectors. Consequently, the internalized DNA is easier to escape from the membrane-disrupted endosome into the cytoplasm and depart from the polyplexes to accomplish biological functions. In comparison, such a degradation-induced improvement is not effective for OEI-GK(1 : 2). Thus the endosomal escape and cytoplasmic release is somewhat hampered, resulting a relatively lower transfection efficiency.



Scheme 2 Schematic illustration of the transfection process of OEI-GKs.

Conclusions

Current developments of polycationic vectors involve two major aims: to generate vectors that mediate a higher transfection efficiency, and to make vectors less toxic, more biocompatible, and biodegradable. The purpose of our study was to develop a desirable gene vector specifically designed according to the unique pH decline in the endosome compartment. OEI800 with negligible toxicity but also poor transfection activity was linked with ketalized glycolic acid to accomplish the enhanced DNA delivery whilst retaining the high cell-biocompatibility. Three kinds of acid-cleavable cationic polymers, termed OEI-GKs, were prepared at different charge ratios, resulting in different architectures and molecular weights of the obtained polymers. By such a design, the multifunction of OEI-GKs was realized in a controllable manner with improved acute cytotoxicity and expected long-term biocompatibility, acid-responsive degradation as well as enhanced buffer capability and relatively easy disassembly of polyplexes after endocytosis.

The OEI-GK(1 : 1) combining the branched structure of OEI800 and the linear structure adopted by OEI-GK was found to potentially act as an efficient gene vector in terms of the transfection activity being even higher than PEI25k as well as negligible cytotoxicity. Although great challenges still remain to probe how acid-triggered degradation in the endosome affects the gene transfection inside the cell by using existing techniques, those encouraging results are believed to be associated with the enhanced buffer capability and decreased DNA binding strength of OEI-GKs upon acid-triggered decomposition in the endosome.

The design of OEI-GKs provides us with a new way to deliver DNA effectively and to remarkably reduce the cytotoxicity usually associated with PEIs. Furthermore, the relatively stable linkage amongst OEI-GK structures besides ketal groups, which is different from the reported acid-cleavable PEI analogues, can help us to develop a useful model for the further in-depth investigation on the relationship between endosome-associated polymer degradation and gene transfection efficacy, thus acquiring more information in future about the transfer process in the endosome.

Acknowledgements

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